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THE FREQUENCY OF ALLELE CCR5Δ32 IN A SERBIAN POPULATION

UČESTALOST ALELA CCR5Δ32 U SRPSKOJ POPULACIJI

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Summary

Background: The mutant CCR5Δ32 allele confers resistance to HIV infection. Several hypotheses regarding its origin and persistence in the human population have been proposed. It is assumed that the Δ32 mutation was introduced in Northern or Eastern Europe and that it spread to the south. Although the frequency of CCR5Δ32 was determined in numerous European nations and regions, further data are needed to complete the puzzle of CCR5Δ32 distribution within the continent.
Methods: To this end, CCR5Δ32 frequency was determined in a Serbian population (sample size 352). DNA was extracted from peripheral whole blood and polymerase chain reaction specific for CCR5 gene was performed. A reaction product of 263 bp was obtained from the wild-type CCR5 sequence and a product of 231 bp was obtained from the truncated CCR5Δ32 sequence.
Results: Overall allele frequency of CCR5Δ32 is 4.55%; 0.57% of individuals in the examined population are homozygous and 8.52% are heterozygous for CCR5Δ32.
Conclusions: The determined frequency of the CCR5Δ32 allele in a Serbian population is unexpectedly low, considering ethnically related populations.

Keywords: CCR5, Δ32, Serbia

Kratak sadržaj

Uvod: Nosioci alela CCR5Δ32 su relativno rezistentni na infekciju HIV-om. Postoji nekoliko hipoteza o poreklu i održanju ovog alela u ljudskoj populaciji. Pretpostavlja se da je mutacija Δ32 nastala u populaciji severne ili istočne Evrope i da se potom proširila ka jugu. Iako je učestalost CCR5Δ32 određena u mnogim evropskim populacijama, dodatni podaci su neophodni za formiranje sveobuhvatne slike o distribuciji CCR5Δ32 u Evropi. Zbog toga smo u našoj studiji odredili učestalost CCR5Δ32 u srpskoj populaciji, za koju do ovog rada nisu postojali takvi podaci.
Metode: DNK je izolovana iz periferne krvi 352 osobe. U reakciji lančanog umnožavanja korišćeni su prajmeri specifični za gen CCR5. Dobijen je proizvod od 263 bp na osnovu matrice »wild type«, sekvence CCR5 gena, a proizvod od 231 bp na osnovu okrnjene sekvence gena CCR5 (CCR5Δ32). Ukupna učestalost alela CCR5Δ32 u srpskoj populaciji iznosi 4,55%.
Rezultati: Od ukupnog broja analiziranih osoba, identifikovano je 8,52% heterozigotnih i 0,57% homozigotnih nosilaca za ovaj alel.
Zaključak: Utvrđena učestalost alela CCR5Δ32 u srpskoj populaciji je neočekivano niska, u poređenju sa učestalošću u ostalim slovenskim populacijama.

Ključne reči: CCR5, Δ32, Srbija

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List of non-standard abbreviations: AIDS, acquired immunodeficiency syndrome; bp, base pair; CFTR, cystic fibrosis transmembrane conductance regulator; HIV, human immunodeficiency virus; n, number; PCR, polymerase chain reaction; SD, standard deviation.

Introduction

Chemokines and their receptors have an essential role in directing cell migration during development and in immunity. However, viruses and other pathogens are able to produce chemokine-like and chemokine receptor-like molecules and to modulate the immune response directed against them (1). As an extreme example, human immunodeficiency virus (HIV) uses chemokine receptors as co-receptors to enter human cells. The most prominent co-receptors for HIV entry into cells are CCR5 and CXCR4 (2). CCR5 is the major co-receptor during primary infection. Importantly, the CCR5 gene is polymorphic and the CCR5Δ32 allele is linked to HIV restriction, viral control, and progression to acquired immunodeficiency syndrome (AIDS) (2). The CCR5Δ32 allele has a 32-base pair deletion within exon 3 in comparison to the wild type allele. Out of these 32 base pairs, 20 base pairs are missing in the coding sequence. Consequently, an mRNA frameshift occurs which creates a stop codon and leads to generation of the truncated CCR5 protein. In this way, HIV binding to CCR5 and entrance of the virus into cells is prevented (2, 3). Hence, homozygotes for the CCR5Δ32 allele exhibit a strong, yet incomplete resistance to HIV infection, while heterozygotes show delayed progression to AIDS (3).

The CCR5Δ32 allele appeared in the European population long before the HIV epidemic. Libert et al. (4) hypothesized that the allele originated in Northeastern Europe and spread towards the south of the continent. An alternative assumption came from Balanovsky et al. who suggested that the allele emerged among Uralic-speaking populations and that its frequency increased in Northeastern Europe as a result of positive selection and/or genetic drift (5). According to this group, secondary spread and selection occurred due to gene flow and migrations. This secondary spread might have been performed by Vikings, as suggested by Lucotte (6). Selective pressure has mainly been attributed to infectious diseases. One of the major candidates has been the bubonic plague that swept across the Europe in the 14th century and killed up to a third of its population (7). However, similar frequency of the CCR5Δ32 allele was determined in ancient DNA samples from individuals that died from bubonic plague and control subjects, arguing against the plague as the selective factor for the allele persistence (8). Other suspected diseases include viral hemorrhagic fever (9) and smallpox (10). Stephens et al. (7) suggested that the allele originated in the European population some 700 years ago, while more recent genetic mapping and studies on ancient DNA indicate that the allele might have been present in human populations for 3–7 thousand years (11). Accordingly, relatively high frequency of the CCR5Δ32 allele was found in ancient DNA samples from medieval Poland (12). Further, the allele was isolated from ancient DNA samples from Bronze Age skeletons found in central Germany and southern Italy (13). Importantly, linkage disequilibrium in the CCR5 region is similar to other human genomic regions, which along with the extent of heterozygosity and differentiation across populations challenges the hypotheses of recent positive selection of the CCR5Δ32 allele (11). Additionally, Faure and Royer-Carenzi (14) have proposed that the allele has not been positively selected, but rather present at low frequency due to the yet unknown zoonosis spread from Mediterranean civilizations northward. If immune defense against such zoonosis is dependent on an intact CCR5 protein, individuals carrying a truncated form of CCR5Δ32 would be vulnerable to the disease. Although individuals homozygous for CCR5Δ32 show no obvious immunodeficiency, it has recently been reported that this allele is a risk factor for both early and late clinical manifestations of the West Nile virus infection (15, 16). Alternatively, there are hypotheses that the frequency of CCR5Δ32 has been influenced by climatic or geographical factors (5, 17).

Here, the frequency of the mutant CCR5Δ32 allele was analyzed in a Serbian population for the first time. Relatively low frequency of the allele in comparison to other Slavic populations was detected.

Materials and Methods

Population

According to previously published data on the frequency of CCR5Δ32 in populations neighboring Serbia, ranging from less than 4% in Greece and exceeding 8% in Hungary (6, 7, 14), it was assumed that the frequency of the allele in a Serbian population would be 6.0 +/–2.5 %. Subsequently, a sample size of 350 was estimated to have a 95% confidence level, as calculated from the following formula:

$$n \geq \left(\frac{z}{m}\right)^2 \times p(1-p)$$

where n is sample size, z is critical value for the confidence level, m is margin of error, p is the supposed proportion of allele. Thus, we examined 352 residents of Serbia in our study. Basic data about the population are as follows: 219 females and 133 males, age (mean +/– SD) 38.9+/–13.3 years, age median 37.0 years, age range 1–83 years.

DNA isolation and amplification

DNA was extracted from peripheral whole blood using GFX Genomic Blood DNA Purification kits (GE Healthcare, Little Chalfont, UK). Polymerase chain reactions were performed in a 25 μmol/L containing: 2 μL of dsDNA template (~100 ng), 2.5 μL of 10X Taq buffer with $(NH_4)_2SO_4$, 2.5 μL of 25 mmol/L $MgCl_2$, 0.2 μL of 25 mM dNTP mix, 1 μL of 10

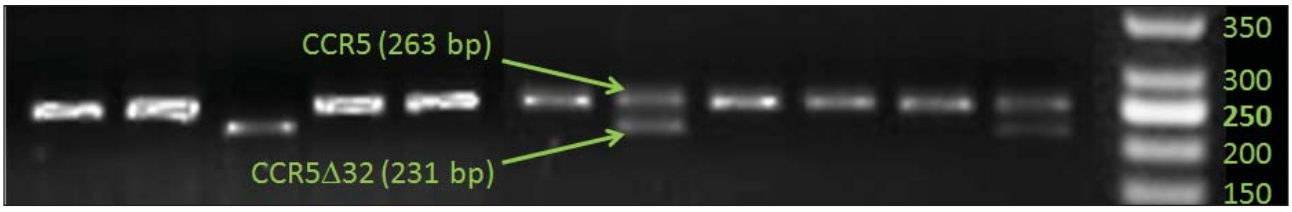

Figure 1 Electrophoresis profile of PCR products obtained with CCR5 amplifying primers. Representative samples are shown. DNA ladder scale is in bp.

μmol/L forward primer (5′-CCC AGG AAT CAT CTT TAC CAG-3′) to a final concentration of 0.5 μmol/L, 1 μL of 10 μmol/L reverse primer (5′-CCC AGA AGA GAA AAT AAA CAA TCA T-3′) to a final concentration of 0.5 μmol/L, 0.2 μL of 5 U/μL Taq polymerase (Thermo Scientific, Waltham, MA) and 15.6 μL of water. The polymerase chain reaction was performed in Thermocycle T1 (Biometra, Jena, Germany). After initial denaturation at 95 °C for 5 min, the amplification was performed for 40 cycles under the conditions: 95 °C for 30, 60 °C for 30 and 72 °C for 1 min. Electrophoresis of samples was performed in 1% agarose gel and PCR products were visualized by ethidium-bromide staining. A PCR product of 263 bp was obtained from the wild-type CCR5 sequence and a PCR product of 231 bp was obtained from the truncated CCR5Δ32 sequence (*Figure 1*).

Statistical analysis

To determine the statistical significance of the difference between the frequency of CCR5Δ32 allele in the Serbian population and other European populations, a two-tailed (H0: p1=p2; Ha: p1≠p2) difference of proportions z-test was performed ($p<0.05$ was considered statistically significant).

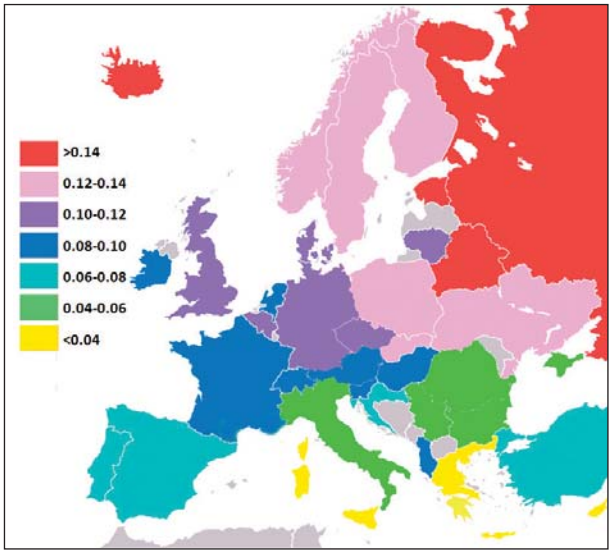

Figure 2 Multicolor representation of CCR5D32 allele frequency in Europe. This hypothetic distribution of the allele frequency was made according to data shown in *Table I* and additional data for Austria and Romania that due to low sample size were not included in *Table I* (Faure et al., 2008). For countries with more than one piece of data, approximations were made for mean frequency. In most cases country boundaries were respected, except for major European islands and Crimea peninsula.

Results and Discussion

Out of 352 analyzed samples, there were two homozygous and 30 heterozygous for the CCR5Δ32 allele. Thus, 0.57% of individuals in the examined population were homozygous and 8.52% were heterozygous for CCR5Δ32. Overall frequency of the CCR5Δ32 allele in the Serbian population was 4.55%. We further determined if this frequency is significantly different to the frequency of CCR5Δ32 allele in other European populations. We had to exclude some of the published data about the CCR5Δ32 allele frequency (e.g. Austria and Romania) from our analysis, as their sample size was not big enough for the test applied. Results of the analysis are shown in *Table I*.

The determined frequency of the CCR5Δ32 allele in the Serbian population is the lowest observed among Slavic populations. The closest proportion of the allele was observed in a Bulgarian population that is geographically closest to Serbia (East–West direction), but with a strong ethnic influence of non-Slavic origin. With Croats, however, who are also close by to Serbs (West–East direction), the difference is larger (*Figure 2*). On the other hand, similar frequencies of the CCR5Δ32 allele were observed in Greece and Romania which are ethnically distant, yet geographically close to Serbia. However, similar frequencies were determined in both ethnically and geographically distant populations (Italy, some parts of Spain and Portugal), as well as in Crimea whose population is ethnically closer to the Serbian.

The observed discrepancy in the CCR5Δ32 allele frequency in Serbian and neighboring populations or ethnically close populations is not an isolated phenomenon. For instance, it was previously reported that the frequency of mutations of the gene encoding the cystic fibrosis transmembrane conductance regu-

Table I Comparison of frequencies of CCR5Δ32 allele in the Serbian population and other European populations.

Country	city, region, nation	frequency	z	Reference
Cyprus	Greeks	0.027	1.782	Faure et al. 2008
Italy	Sardinia	0.027	1.563	Faure et al. 2008
France	Corsica	0.032	0.899	Faure et al. 2008
Greece	Crete	0.032	0.892	Faure et al. 2008
Spain	Sevilla	0.038	0.395	Faure et al. 2008
Greece		0.039	0.542	Faure et al. 2008
Italy	Sicily	0.040	0.439	Faure et al. 2008
Greece	Athens	0.041	0.171	Magierowska et al. 1998
Greece	Greeks	0.044	0.086	Stephens et al. 1998
Serbia		0.046	0.000	
Italy	Italians	0.047	–0.140	Faure et al. 2008
Italy	Padua	0.047	–0.024	Faure et al. 2008
Spain	San Sebastian	0.050	–0.191	Magierowska et al. 1998
Ukraine	Crimea	0.050	–0.402	Faure et al. 2008
Bulgaria		0.051	–0.112	Faure et al. 2008
France	Nice	0.052	–0.134	Faure et al. 2008
Portugal	Porto	0.052	–0.134	Faure et al. 2008
Turkey		0.054	–0.320	Faure et al. 2008
Italy	Italians	0.055	–0.346	Stephens et al. 1998
Italy	Rome	0.057	–0.735	Faure et al. 2008
Spain	Basques	0.062	–0.850	Stephens et al. 1998
Turkey	Turks	0.063	–0.895	Libert et al. 1998
Portugal	Lisbon	0.064	–0.575	Faure et al. 2008
Portugal	Portuguese	0.064	–0.575	Libert et al. 1998
Croatia	Island of Rab, Lopar	0.065	–0.640	Faure et al. 2008
France	Perpignan	0.068	–0.940	Faure et al. 2008
Spain	Basques	0.068	–0.954	Faure et al. 2008
Croatia		0.071	–1.482	Faure et al. 2008
Spain	Spanish	0.073	–1.030	Faure et al. 2008
Spain	Catalonia	0.074	–1.360	Faure et al. 2008
Slovenia	Slovenians	0.077	–1.125	Stephens et al. 1998
Sweden	Lapland	0.080	–1.571	Faure et al. 2008
Albania		0.082	–1.289	Faure et al. 2008
Ireland		0.083	–1.810	Faure et al. 2008
Slovenia		0.083	**–2.140**	Faure et al. 2008
Sweden	Saamis	0.083	–1.571	Libert et al. 1998
Switzerland	Bern	0.085	–1.098	Faure et al. 2008
Hungary	Hungarians	0.086	–1.746	Libert et al. 1998
France	Reims	0.087	**–2.114**	Faure et al. 2008
Italy	Milano	0.087	–1.773	Libert et al. 1998
Italy	Milan	0.087	–1.773	Faure et al. 2008
France	French	0.089	**–2.032**	Faure et al. 2008
France	French	0.089	**–2.032**	Stephens et al. 1998
Ukraine	Lugansk	0.090	–1.738	Faure et al. 2008
Finland	Finns	0.091	**–2.174**	Stephens et al. 1998
Belgium	Belgians	0.092	**–2.698**	Libert et al. 1998

Spain	Murcia	0.095	**–2.067**	Libert et al. 1998
Spain	Murcia	0.095	**–2.067**	Faure et al. 2008
Spain	Oviedo	0.096	**–2.454**	Faure et al. 2008
Ukraine	Lvov	0.096	–1.679	Faure et al. 2008
Spain	Spanish	0.098	–1.379	Stephens et al. 1998
France	Montpellier	0.099	**–2.818**	Faure et al. 2008
Netherlands		0.100	**–2.755**	Faure et al. 2008
France	Montpellier	0.101	**–2.095**	Libert et al. 1998
Czechia	Czechs	0.102	**–2.344**	Stephens et al. 1998
Hungary	Hungarians	0.104	–3.029	Faure et al. 2008
Norway	Norwegians	0.105	**–2.403**	Libert et al. 1998
Germany	Mulheim	0.106	**–2.095**	Faure et al. 2008
Czechia		0.107	**–3.303**	Faure et al. 2008
Lithuania	Vilnius	0.107	**–2.926**	Faure et al. 2008
France	Lille	0.108	**–2.374**	Faure et al. 2008
Germany	Germans	0.108	**–2.742**	Faure et al. 2008
Denmark	Danes	0.110	**–2.403**	Libert et al. 1998
France	Brittany	0.110	**–2.403**	Libert et al. 1998
Ukraine	Kiev	0.110	**–2.218**	Faure et al. 2008
France	Nancy	0.111	**–3.098**	Faure et al. 2008
Hungary	Budapest	0.111	**–2.240**	Magierowska et al. 1998
Great Britain	Brits	0.115	**–3.685**	Faure et al. 2008
Lithuania	Lithuanians	0.115	**–3.340**	Libert et al. 1998
Finland	Finns	0.116	**–3.381**	Faure et al. 2008
Great Britain	British	0.117	**–3.529**	Stephens et al. 1998
Denmark	Danes	0.118	**–3.574**	Faure et al. 2008
Poland		0.118	**–3.935**	Faure et al. 2008
Belgium	Leuven	0.119	**–3.496**	Faure et al. 2008
Norway	Oslo	0.120	**–3.194**	Faure et al. 2008
Russia	Ryazan	0.120	**–2.388**	Faure et al. 2008
Russia	Tatar	0.120	**–2.169**	Stephens et al. 1998
France	Paris	0.122	**–3.657**	Faure et al. 2008
France	Brest	0.123	**–3.298**	Faure et al. 2008
Estonia	Estonians	0.133	**–3.521**	Stephens et al. 1998
Russia	Russians	0.136	**–2.694**	Stephens et al. 1998
Sweden	Swedish	0.137	**–3.512**	Stephens et al. 1998
Norway	Oslo	0.138	**–2.958**	Magierowska et al. 1998
Russia	Moscow	0.138	**–3.459**	Faure et al. 2008
Russia	Russians	0.139	**–3.310**	Libert et al. 1998
Slovakia		0.140	**–4.305**	Faure et al. 2008
Sweden	Stockholm	0.140	**–4.105**	Faure et al. 2008
Sweden	Umea	0.142	**–4.029**	Libert et al. 1998
Sweden	Stockholm	0.143	**–3.243**	Magierowska et al. 1998
Estonia		0.144	**–4.761**	Faure et al. 2008
Iceland		0.147	**–3.582**	Faure et al. 2008
Poland	Poznan	0.155	**–3.236**	Magierowska et al. 1998
Finland	Finns	0.158	**–3.720**	Libert et al. 1998
Belarus	Belarusians	0.160	**–3.776**	Faure et al. 2008
Russia	Mordvinians	0.163	**–3.862**	Libert et al. 1998
Russia	St. Petersburg	0.166	**–2.565**	Magierowska et al. 1998

Frequencies of CCR5Δ32 allele in various European populations. Z values that reach statistical significance using a 95% confidence level are written in bold. Data are taken from the papers indicated under the references.

lator (CFTR), including F508del, 5T and M470V, differs significantly in the Serbian population in comparison with other South European populations (19–21). It would be important to obtain data on the frequency of the CCR5Δ32 allele in Bosnia, Herzegovina, Montenegro and Macedonia to get further insight into the ethno-geographical relation of the CCR5Δ32 allele frequency in southeastern Europe.

Finally, besides the importance that our results have for studies of the origin and spread of CCR5Δ32 allele in European populations, they are also important for clinical practice. It has recently been reported that the cure of HIV has been achieved in a patient grafted with CCR5Δ32/Δ32 stem cells (22). Thus, low frequency of CCR5Δ32 homozygous individuals indicates that there will be less potential donors of CCR5Δ32/Δ32 stem cells in the Serbian population.

The ethnical map of Europe is even more complex than its political map and there has been intensive blending of European populations (both within the continent and with populations from other continents) due to vivid historical events and processes (migrations, assimilations, international marriages). Thus, further determination of CCR5Δ32 frequency in various regions and ethnic groups of Europe and analysis of data so obtained in conjunction with geographical and historical facts are needed in order to construct a definitive map of the CCR5Δ32 allele frequency in Europe. Further, intensive studies of ancient DNA are imposed. Finally, the CCR5Δ32 allele frequency has to be investigated in parallel with haplogroup analyses. Only then we might be able to give answers on the origin and spread of the allele in the European population.

In conclusion, CCR5Δ32 frequency is relatively low in the Serbian population in comparison to other Slavic nations. Yet, it is similar to the frequencies determined in some populations of southern and southeastern Europe.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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